I. Introduction

Over the past few years, the fundamental roles of STATs in highly diverse biological processes are being increasingly recognized. It is now known that many cytokines, hormones and growth factors utilize STAT signaling pathways to control a remarkable variety of biological responses, including development, differentiation, cell proliferation and survival. Ligand-activated cytokine or growth factor receptors initiate STAT signal transduction either by the activation of the Jak family of tyrosine kinases (TKs) or via intrinsic receptor tyrosine kinase domains, which results in phosphorylation of tyrosine residues of STATs. Also, cytoplasmic tyrosine kinases other than Jaks have now been identified which activate STATs. It is therefore more appropriate to designate signaling pathways that utilize STAT transcription factors as TK-STAT pathways rather than originally termed Jak-STAT pathways. Tyrosine phosphorylation of STATs allows STAT dimerization, nuclear translocation and binding of response elements in the promoters of target genes. Additional modifications, including the phosphorylation of serine residues, are required to achieve maximal transcriptional activity. It is currently being appreciated that STATs function in multi-protein enhanceosomes to initiate gene transcription. The association of STATs with a variety of nuclear proteins has now been described, including associations of STATs with bridging co-activator proteins which couple activated STATs to the basal polymerase II transcription machinery. Since STAT proteins play critical roles in such a broad spectrum of cellular processes, it is not surprising that inappropriate activation of STAT signaling pathways are also increasingly associated with oncogenesis. Indeed, there is mounting evidence that constitutively-activated STATs participate in cell transformation induced by numerous oncogenes and in malignant progression of human cancers.

II. The TK-STAT pathway

II-1. Cytokine and Growth factor receptors

STATs are activated in response to a variety of cytokines and growth factors, each initiating signal transduction by association with their appropriate receptor (Table 1) [1]. These receptors can be subdivided into two groups on the basis of patterns of conserved amino acids within the extracellular domain, termed class I and class II receptors [2]. Class I includes receptors for IL-2, IL-3, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15, Epo, PRL, GH, G-CSF, GM-CSF, LIF, CNTF and thrombopoietin [1]. These receptors share conserved motifs containing cysteine and tryptophan residues in their extracellular N-terminal region. A typical sequence of tryptophan-serine-X-tryptophan-serine (WSxWS), where X refers to any non-conserved amino acid residue, is involved in cytokine binding [3]. Class II receptors share overall structural features, but are more divergent. Class II receptors, which include the receptors for IFN-α, IFN-γ and IL-10, encode an additional conserved cysteine pair, and several conserved proline and tyrosine residues [4,5]. Importantly, class I and II receptors function as multiprotein complexes and in some cases receptor components are shared amongst cytokine receptors, providing an explanation for the remarkable redundancy in biological functions that is sometimes observed amongst several cytokines.

The cytokine receptor family has been further sub-divided based on the characteristic structural motifs in their extracellular domains [1,6]. The main sub-types of this family are (a) the gp130 family, (b) the IL-2 family, (c) the growth hormone family, (d) the interferon family, (e) the gp140 family and (f) the receptor tyrosine kinase family (Table 1). Each receptor family is activated by a specific sub-set of ligands (Table 1). The gp130 family will be discussed below and for detailed information on the other cytokine receptor families readers are referred to some expert reviews on this topic [6-9].

Table 1. Activation of JAK kinases and STATs by various ligands (adapted from [1]).

	JAKS				STATs					
Ligands	Jak1	Jak2	Jak3	Tyk2	Stat1	Stat2	Stat3	Stat4	Stat5	Stat6
gp130 family										
IL-6	+	+	-	+	+	-	+	-	-	-
IL-11	+	+			+	-	+	-	-	-
OnM	+	+	+	+	+	-	+	-	-/+	-
LIF	+	+			+	-	+	-	-/+	-
CNTF	+	+		-/+	+	-	+	-	-	-
IL-12	-	+	-	+	+	-	+	+	-	-
IL-2 family										
IL-2	+	-	+	-	+/-		+		+	
IL-4	+	-	+	-						+
IL-7	+	-	+	-					+	
IL-9	+	-	+	-					+	
IL-13	+			-						+
IL-15	+		+						+	
Growth hormone family										
EPO	-	+	-		+	-	+	-	+	
GH	-	+	-		+	-	+	-	+	
PRL	+/-	+	-		+	-	+	-	+	
G-CSF	+	+			+	-	+	-	+	
IFN family										
IFN-α	+	-	-	+	+	+	+	-	+	
IFN-β	+	-	-	+	+	+				
IFN-γ	+	+	-		+	-	-	-		
IL-10			-	+	+/-	-	+	-		
gp140 family										
IL-3	-	+	-	-					+	
IL-5	-	+	-	-					+	
GM-CSF	-	+	-	-	+		+		+	
Receptor tyrosine kinases										
EGF	+	+			+	-	+	-	+	
PDGF	+	+			+	-	+	-	+	
CSF-1	+	+			+	-	+			

II-2. The gp130 receptor family

Gp130 is the common subunit of the receptor complexes for the IL-6 family of cytokines, which includes LIF, CNTF, OnM, IL-6, IL-11, and CT-1 [10]. As a consequence, these cytokines elicit similar and overlapping physiological responses and are therefore referred to as the 'IL-6-type cytokines'. The receptors involved in IL-6-type cytokine signaling identified so far consist of a ligand-binding α-chain and a signal transducing chain, except for LIF and OnM, which bind their corresponding signal transducing chains directly. IL-6-type cytokine receptors belong to the receptor class I family, which is defined by the presence of at least one cytokine-binding module (CBM) consisting of two fibronectin-type-III-like domains of which the N-terminal domain contains a set of four conserved cysteine residues and the C-terminal domain a WSxWS motif (Fig.1) [3]. All receptors contain an IgG like domain and the signal transducing chains have three additional membrane-proximal fibronectin-type-III-like domains (Fig.1) [3].

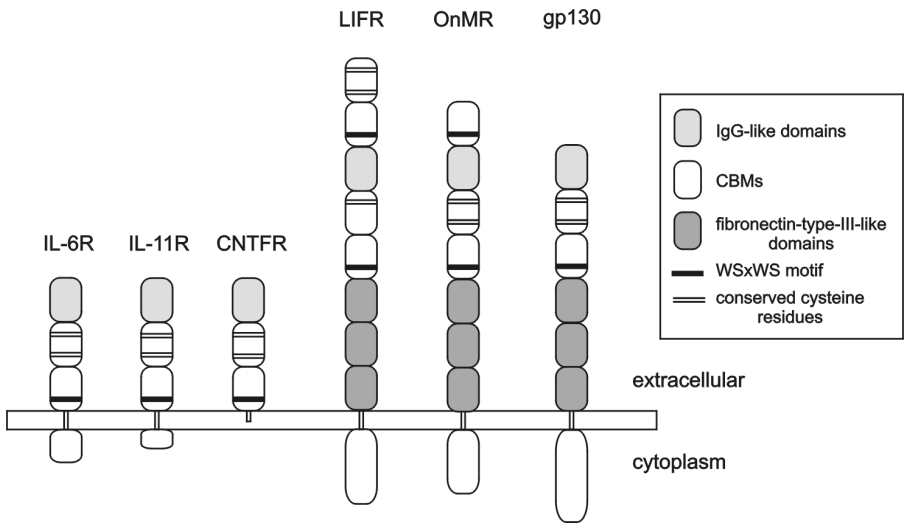

Figure 1. Domain composition of receptors involved in IL-6-type-cytokine signaling. (adapted from [10], CBM = cytokine binding module).

Studies using transgenic mice expressing the genes encoding mutated IL-6-type cytokines or gp130 have revealed that gp130-mediated signals are involved in the immune, hematopoietic, nervous, cardiovascular, and endocrine systems, as well as in bone metabolism, inflammation, the acute phase response, plasma cell proliferation, osteoporosis, liver regeneration, and hepatocyte maturation [10,11]. Binding of IL-6-type cytokines to their receptors leads to homodimerization of gp130 (in the case of IL-6 and possibly IL-11 [12]) or heterodimerization of gp130 with other gp130 related receptors (in the case of LIF, CNTF, OnM and CT-1 [13-15]). IL-6, IL-11 and CNTF first bind to their specific α-receptor subunits (designated as IL-6R, IL-11R and CNTFR) which are not involved in the intracellular signal transduction cascade. Upon ligand binding the

complexes of cytokine and α-receptor efficiently recruit the corresponding signal-transducing components. LIF and OnM directly bind their signal transducing chains (the LIFR and OnMR, respectively) in order to induce dimerization with gp130. In some cases, the membrane-bound α-receptors can be functionally replaced by soluble forms, which lack transmembrane and cytoplasmic regions that are generated by translation of alternatively spliced RNAs [16,17]. Soluble forms of both gp130 (sgp130) and the IL-6R (sIL-6R) have been identified [18]. The sIL-6R might serve as an agonist, whereas sgp130 possibly acts as an antagonist by neutralizing IL-6-sIL-6R complexes, although the exact physiological role of these soluble receptors still needs to be elucidated.

II-2a. IL-6-gp130 signaling

Upon binding of IL-6 to the IL-6R, the gp130 signal transduction chain is recruited to this complex. Information from ternary-complex-formation assays suggest that the activated receptor complex is hexameric consisting of two molecules of IL-6, IL-6R and gp130 molecules [19,20], although further experiments are required to confirm this model. Upon activation, multiple tyrosine residues of the gp130 chain become phosphorylated, which form docking sites for various signal transduction molecules. The known signal transduction cascades that are activated by gp130 are the TK-STAT pathway, the Ras-ERK pathway, the PI-3K pathway and a signal transduction cascade that is initiated by Vav. The structural features of gp130 are summarized in Fig.2. However, the kinetics and mechanisms of activation of these signal transduction cascades are highly dependent on the cell-type.

Association of Jaks with the receptor is thought to be mediated by the membrane-proximal box1/box2 regions that are conserved among many cytokine receptors [21,22]. Box1 is a proline rich motif of eight amino acid residues, while box2 is a cluster of hydrophobic amino acid residues followed by positively charged amino acids. IL-6 leads to the activation of Jak1, Jak2 and Tyk2 [1]. In some cell types other tyrosine kinases are activated as well, including Hck, Fes, Btk and Tec, although their exact physiological role in IL-6 signaling remains to be determined [23-25]. It is assumed that Jaks are constitutively associated with gp130 monomers, and that upon IL-6 stimulation and gp130 dimerization, Jaks can transphosphorylate the tyrosine residues 759, 767, 814, 905 and 915 of the associated gp130 receptor component (Fig.2) [10]. Phosphorylation of the tyrosine residues 767, 814, 905 and 915, which are located in conserved YxxQ boxes, creates docking sites for the transcription factors STAT1 and STAT3 [26,27]. STAT association with the gp130 receptor results in Jak-mediated tyrosine phosphorylation of STATs, which enables STAT dimerization and nuclear translocation. The mechanism of IL-6-induced STAT3 activation will be discussed in detail in part II-5. Phosphorylation of the tyrosine residue 759 is thought to recruit the tyrosine phosphatase SHP2, which is subsequently phosphorylated by Jaks [27,28]. Although SHP2 downstream signaling is not well defined, mutation of tyr759 reduces the interaction between SHP2 and Grb2 and between SHP2 and Gab, and lowers the gp130-induced activation of ERK MAP kinases, suggesting that SHP2 mediates signals to the ERK cascade through Grb2 and Gab proteins [29-31].

PI-3K is activated in response to IL-6 stimulation via the gp130 receptor [32]. The mechanism of the IL-6-induced PI-3K activity is not completely established but it appears that Jak1 can associate with PI-3K and subsequently phosphorylates the p85 subunit of PI-3K [33].

The guanine nucleotide exchange factor Vav is also associated with the gp130 receptor, and IL-6 stimulation results in a transient tyrosine phosphorylation and activation of Vav [34]. The Vav binding site has been mapped in the membrane-distal region of gp130, and Vav activation initiates signaling via the Ras signal transduction cascade by association with Grb-2 [34].

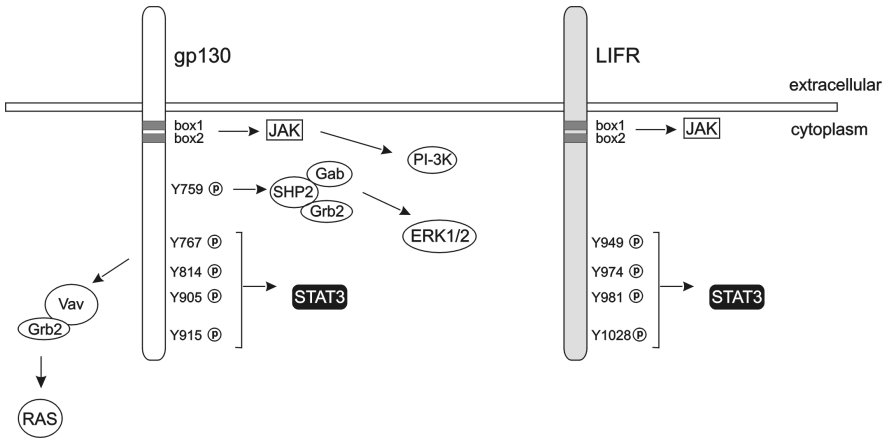

Figure 2. Functional domains and signal transduction cascades that are activated by the gp130 and LIF receptor.

II-2b. LIF-gp130 signaling

LIF induces heterodimerization of gp130 with the LIFRβ, as has also been described for CT-1, CNTF and probably OnM. While IL-6 first binds to its specific α-receptor subunit in order to recruit gp130, LIF binds directly to its signal transduction components. The LIFRβ contains box1 and box2 membrane proximal regions that are involved in Jak binding, as is the case for gp130. Also, four STAT3 docking sites are present in the LIFRβ, located at tyrosine residues 949, 974, 981 and 1028.

II-3. The Janus kinase family of tyrosine kinases

Jaks are intracellular tyrosine kinases with molecular masses of 120-140 kDa (Fig.3A). Four members have now been identified in mammalian cells, Jak1, Jak2, Jak3 and Tyk2. Jak3 is mainly expressed in cells of hematopoietic origin, while the other Jaks are widely expressed. Jaks consist of 7 Jak homology (JH) domains, which share a high sequence similarity. At the C-terminus, they contain a typical tyrosine kinase domain (JH1), which is preceded by a kinase-like domain (JH2). Within the kinase domain, Jaks share considerable sequence similarity with other tyrosine kinases with respect to an activation loop implicated in the regulation of kinase activity (reviewed in [21,35]). Two tyrosine residues within the activation loop of Tyk2 have been found to be phosphorylated, and mutation of these residues eliminates essentially all tyrosine kinase activity [36]. The significance of the tyrosine-like domain is less clear. Deletion of this region in Tyk2 leads to a protein that is no longer able to phosphorylate an exogenous substrate, suggesting that this domain is required for catalytic activity [37]. However, deletion of the kinase-like

domain of Jak2 did not affect its kinase activity [38]. A negative regulatory function of this domain has been suggested by the observation that a mutation in the kinase-like domain of the Drosophila Jak homologue hopscotch leads to a hyperactive enzyme [39]. Also, mutation of the corresponding residue in Jak2 increased its catalytic activity. The mechanisms that underlie these observations will still have to be elucidated. The N-terminal region of Jaks (JH3-JH7) is involved in receptor association.

II-4. The STAT family of transcription factors

Seven mammalian STATs have now been identified, STAT1, 2, 3, 4, 5A, 5B, and 6 (Fig.3B) [1,21,40,41]. They are localized in three chromosomal clusters suggesting that this family of transcription factors has evolved by gene duplication [42]. Except for STAT2, alternatively spliced isoforms have been described, designated as STAT1α and β, STAT3α, β and γ, STAT5Aα and β, and STAT5Bα and β [43-46]. Although alternatively spliced RNAs have been found for STAT4 and STAT6, the corresponding proteins have not been identified yet [10]. STATs are ubiquitously expressed, except for STAT4, which expression is more restricted to myeloid cells and testis [47]. The regulation of STAT activity is mainly regulated by post-translational modifications, rather than at the level of gene transcription. However, sequences in the 3' untranslated region of the STAT3 gene have been identified as important modulators of RNA splicing which determine the balance between α and β isoforms [48]. How the generation of isoforms of other STATs is regulated is less well understood.
All STATs consist of approximately 750-850 amino acids and have several conserved domains that are critical for STAT functions, including a DNA binding domain, an SH2 domain which is connected to the DNA binding domain via a linker region, a C-terminal transactivation domain and an N-terminal that contains a leucine-zipper-like region and a tetramerization domain (Fig.3B). By means of x-ray crystallography, structural information on STAT1, STAT3 and STAT4 has become available since 1998 [49-51].

II-4a. The DNA binding domain
The DNA binding region has been suggested to regulate DNA binding specificity and its primary structure is not related to any other known protein sequence [52]. However, there is a striking structural and functional similarity between the DNA binding regions of STAT1, STAT3, and the Rel/NF-κB transcription factors which all contact the DNA by using loops protruding from β-barrel domains with a variant of the immunoglobulin variable fold [49,50]. All STATs bind to similar DNA elements (TTN_5AA), which is probably due to the highly conserved amino acid sequences of the DNA binding domains [53,54]. However, systematic analysis of binding of activated STATs to synthetic oligonucleotides showed differences in binding affinity between STAT members [54,55]. These experiments demonstrated that the spacing between the palindromic TT-AA core influences the selectivity of STAT proteins. Sequences with 5 bp between these palindromic half sites are found in many promoters most of which are bound by several STAT complexes, although some display a preference towards specific STAT members. In some promoters, the palindromic TT-AA is separated by 6 bp, which results in the selective binding of STAT6 dimers, while a spacing of 4 bp appears to preferentially allow STAT3 dimer binding. Furthermore, specific DNA binding might depend on the

composition of STAT dimers, since STAT1-STAT3 heterodimers appear to bind different response elements than STAT1 or STAT3 homodimers in some cases [55].

II-4b. The SH2 domain

The function of the SH2 domain is well established. This domain is responsible for the binding of STATs to tyrosine phosphorylated receptor motifs and also for homo- and heterodimerization with other STAT molecules. Upon ligand stimulation, Jak mediated phosphorylation of tyrosine STAT3 docking sites allows the recruitment of STATs to the receptor and the subsequent Jak mediated tyrosine phosphorylation of STATs. The STAT tyrosine phosphorylation site is located around amino acid position 700 (tyr701 for STAT1 and tyr705 for STAT3) and enables STAT dimerization via reciprocal interaction of each STAT SH2 domain. The negatively charged phosphate on the tyrosine residue located at the C-terminal end of the SH2 domain is stabilized by a positively charged arginine residue located at the N-terminal end of the SH2 domain of the partner STAT molecule. Mutation of either tyrosine or arginine residues completely abolishes STAT dimerization (reviewed in [1,10,40,41]).

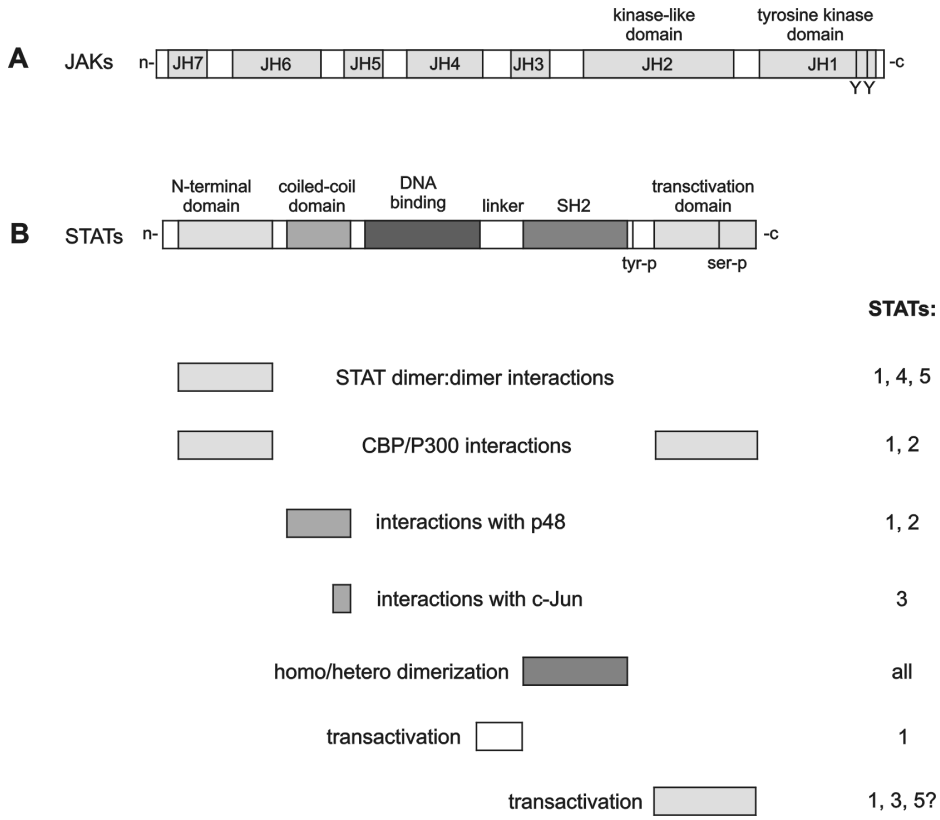

Figure 3. A, Functional domains of JAKs. B, Functional domains of mammalian STATs. (modified version of [67]).

II-4c. The linker region

Based on sequence similarity it was first suggested that the region between the DNA binding domain and the SH2 domain was a functional SH3 domain, possibly involved in mediating interactions with other proteins. However, based on structural information of STAT1 and STAT3, it is now clear that this region is not a functional SH3 domain. This region is now referred to as the linker region of which no specific functions have been identified sofar. Possibly, this region is important for the regulation of STAT transcriptional activity, since mutations in the linker region of STAT1 led to a protein that can be phosphorylated on tyrosine, dimerize, accumulate in the nucleus and bind DNA but fail completely to activate transcription [56].

II-4d. The N-terminal region

The N-terminal region of STATs is required for transcriptional activation as judged by defective stimulation in transfection experiments of NH2 terminal deletion mutants of STAT1 [57]. Also, interactions of the histone actetyltransferases CBP/P300 with the N-terminus of STAT1 have been described which may contribute to transcriptional activation [58]. In addition, many promoters contain closely spaced tandem STAT binding sites. When two such sites are occupied STAT dimer-dimer interactions exist, which are mediated via the N-terminus [51,59]. Dimer-dimer interactions enhance transcriptional activity, especially in the case of STAT5 [60].

II-4e. The C-terminal transactivation domain

The C-terminal region of STATs functions as a transcription activation domain (TAD). STAT1, STAT3 and STAT4 all share a conserved stretch of amino acids in their C-terminal region (LPMSP) in which both the leucine as well as the serine residues are important to achieve maximal transactivational activity [1,61,62]. Upon cytokine or growth factor stimulation, the serine residue becomes phosphorylated, and it has been well documented that this phosphorylation event is critical in order to initiate high levels of gene transcription in various cellular settings [63]. STAT1β and STAT3β, which lack a C-terminal region that contains the LPMSP motif due to splicing events display reduced transcriptional activities [43,64]. Interaction of the C-terminal region of both STAT1 and STAT2 with the histone acetyltransferase CBP/P300 has been described [58], as well as an interaction of STAT1 with the mini chromosome maintenance protein (MCM)-5 which critically depends on phosphorylation of the serine residue 727 [65].

II-4f. The coiled-coil domain

The stretch of amino acids between the N-terminal domain and the DNA binding domain of STATs consists of a coiled-coil structure containing four long helical coils in which interactions with other proteins occur [66]. It mediates the interaction of STAT1 and STAT2 with p48, which belongs to the Interferon Regulatory Family (IRF) of proteins and is required for DNA binding and transcription activation of the STAT1:STAT2:p48 complex (ISGF3) [67]. Furthermore, the transcription factor c-Jun associates with the coiled-coil structure of STAT3, thus strongly enhancing STAT3 driven gene transcription [68]. A recent report by Zhang et al indicated that the coiled-coil domain is also important for early events in STAT3 signaling. It was demonstrated that the coiled-coil domain is essential for Stat3 recruitment to the receptor and the subsequent tyrosine phosphorylation and tyrosine phosphorylation-dependent activities, such as dimer formation, nuclear

translocation and DNA binding, in response to EGF or IL-6 [69]. Single mutation of Asp170 or, to a lesser extent, Lys177 in alpha-helix 1 diminished both STAT3 receptor binding and STAT3 tyrosine phosphorylation.

II-4g. Nuclear import and export

Only recently, the mechanisms that are involved in nuclear import of STATs have become unraveled. Although the interferon-induced nuclear import of STATs is mediated by the importin/Ran system, no classical nuclear localization signal (NLS) has been found in STATs. Melen et al. demonstrated that a structural arginine/lysine-rich stretch in the DNA binding region of STAT1 and STAT2 is required for nuclear import [70]. Two of these arginine/lysine-rich stretches, one in each of the monomers, mediate nuclear import, since dimers consisting of one wild-type STAT and one with mutations in the arginine/lysine-rich stretch did not translocate to the nucleus upon stimulation, thus functioning as a dominant negative. Since the arginine/lysine-rich elements are conserved throughout STAT1, STAT2, STAT3 and STAT4, and information based on the 3D-structures of STAT1 and STAT3, it is plausible that this mechanism of nuclear import is also applicable to other STATs. STAT1 also contains a nuclear export signal (NES), which is located immediately adjacent to the NLS (amino acids 400-409) [71]. STAT1 nuclear export is Leptomycin B (LMB)-sensitive and is regulated by the CRM1 export protein. Whether NESs also exist in other STATs remains to be elucidated.

II-5. IL-6-induced STAT3 activation

II-5a. IL-6

Interleukin-6 (IL-6) is a pleiotropic cytokine that mediates a variety of functions in different cells and tissues including proliferation and differentiation of hematopoietic cells, induction of the acute phase response in liver cells and inflammation at sites of tissue injury [72,73]. It is expressed by many different cell types, including T- and B-cells, monocytes, fibroblasts, keratinocytes, endothelial cells, astrocytes and mesangial cells [11,73,74]. The promoter of IL-6 contains sites for four inducible transcription factors, which includes sites for cyclic AMP Responsive Element Binding protein (CREB), Nuclear Factor IL-6 (NF-IL6), Activator Protein-1 (AP-1) and Nuclear Factor kappa B (NF-κB) [75-77]. IL-6 knockout mice display normal embryogenesis and lymphoid development, but have a disturbed bone metabolism, impaired anti-viral responses, no LPS-induced acute phase response and defects in liver regeneration [10].

II-5b. IL-6-induced STAT3 signaling

IL-6 initiates its action by binding to its α-receptor (IL-6R) which in turn recruits the gp130 receptor to form the activated gp130 receptor complex (Fig.4) [1]. Jaks (Jak1, Jak2, or Tyk2 in the case of IL-6 signaling) are thought to be constitutively associated with monomeric gp130 receptor chains and transphosphorylate tyrosine residues 759, 767, 814, 905 and 915 of the associated gp130 chain upon dimerization [10,26,27]. These phosphorylated tyrosine residues form docking sites for SHP2 (tyr759) or STAT3 (tyr767, tyr814, tyr905 and tyr915) [28,78]. It has been demonstrated that each of these tyrosine STAT3 docking sites contributes significantly to IL-6-induced STAT3 activation. Upon STAT3 association with gp130, Jaks phosphorylate STAT3 on tyr705, which is located in

the conserved SH2 domain. STAT3 tyr705 phosphorylation allows STAT3 dimerization, nuclear translocation and binding of response elements in promoters of target genes [1]. Besides tyr705 phosphorylation, STAT3 is phosphorylated on a serine residue at position

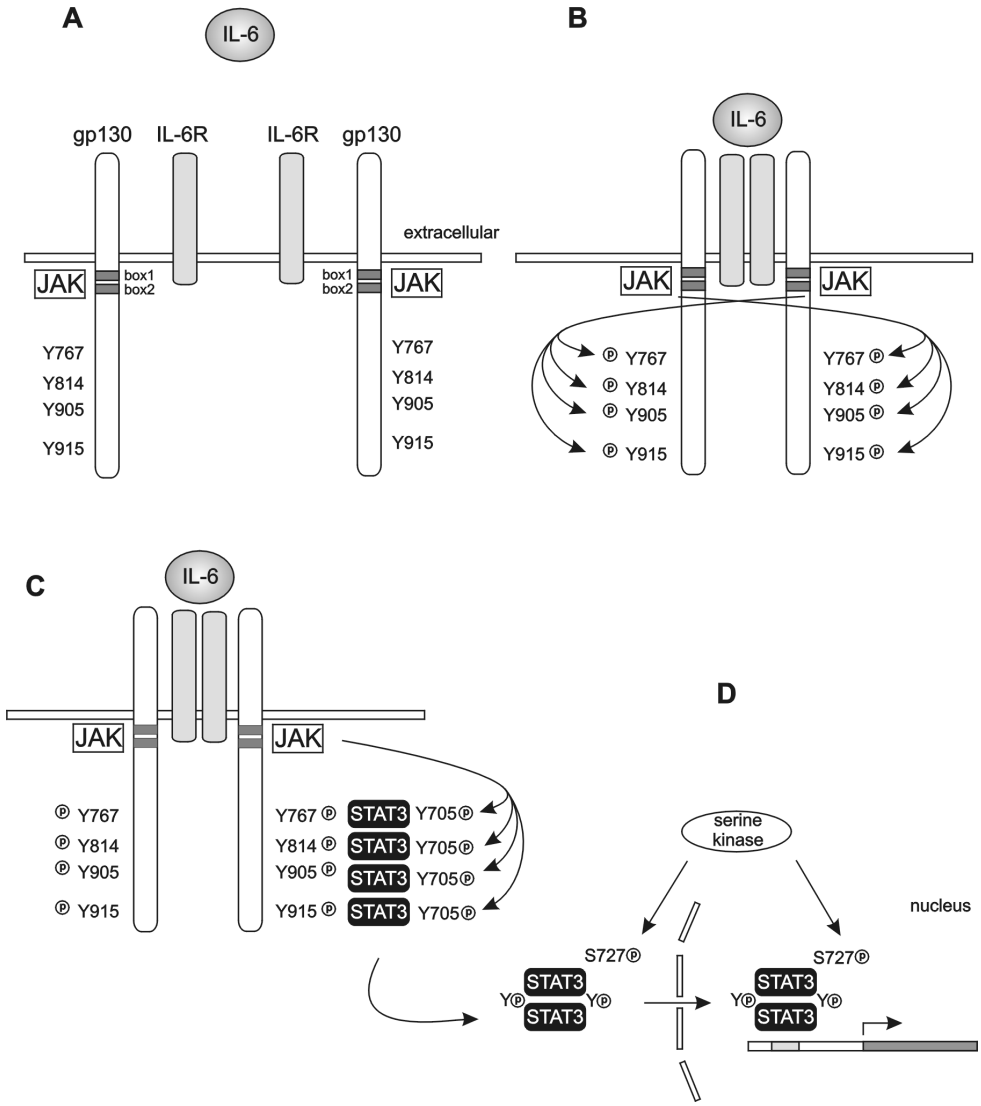

Figure 4. Current model of IL-6 signaling via the gp130/JAK/STAT pathway. A, JAKs are constitutively associated with monomeric gp130 receptor components. **B**, IL-6 binding induces receptor dimerization. JAKs transphosphorylate tyrosine residues in the cytoplasmic part of gp130. **C**, Phosphorylated tyrosine residues function as docking sites for STAT3. STAT3 associates with gp130 and is phosphorylated by JAKs on tyr705. **D**, STATs form dimers and translocate to the nucleus where they regulate gene transcription. In addition, STAT3 is phosphorylated on ser727.

727, which is a prerequisite for maximal STAT3 transactivation in most cellular settings [63]. STAT3 ser727 phosphorylation will be discussed in paragraph II-5d.
A peculiar finding was reported by Ndubuisi et al., who showed that there is a limited amount of monomeric STAT3 in the cytoplasm of unstimulated liver cells [79]. Rather, the bulk of STAT3 was present in the cytosol as high molecular mass complexes in two broad distributions in the size range of 200-400 kDa (designated as 'statosome I') and 1-2 MDa (designated as 'statosome II'). Upon IL-6 stimulation, (i) tyrosine phosphorylated STAT3 was detected both in stratosome I and II, (ii) a small pool of monomeric STAT3 in the range of 80-100 kDa was detected, and (iii) most of the cytoplasmic DNA-binding competent STAT3 was detected in 230 kDa complexes which also consisted of the protein catalase. In a first approach to identify the proteins that are present in the 200-400 kDa statosome I, one protein could be identified as the chaperone GRP58/ER-60/ER75. This chaperone protein associated with STAT3 in an IL-6 dependent manner. These observations indicate that the bulk of cytosolic STAT3 exists in protein assemblies of high molecular mass and suggest that the initiation and transmission of STAT3 signaling through the cytosolic compartment involves novel regulatory, scaffolding, and chaperone proteins, which exact mechanisms still need to be elucidated.
Among the IL-6 target genes are (1) APP proteins such as C-reactive protein, α1-antichymotrypsin, α2-macroglobulin, lipopolysaccharide-binding protein and tissue inhibitor of metallo-proteinases (TIMP)-1; (2) transcription factors such as Jun B, c-Fos, c-Jun, interferon regulatory factor (IRF)-1, MYC, GFAP, STAT3 and CCAAT enhancer binding protein (C/EBP)δ; (3) cell cycle related genes such as cyclin D1, cyclin D2, cyclin D3, cyclin E, cdc25A, p21; (4) apoptosis-related genes such as Bcl2 and Bcl-xL; (5) genes involved in negative feedback loops such as Socs-1 and Socs-3; and (6) a variety of other genes such as interstitial collagenase, vasoactive intestinal peptide, pro-opiomelanocortin, heat-shock protein hsp90, ICAM-1, fibrinogen, OnM, IL-10, and the IL-6 signal transducer gp130. Recent advances in micro-array analysis have revealed new groups of IL-6 and STAT3 responsive genes and information on all the genes that are activated by STAT3 will probably become available in the coming few years. An overview of a number of STAT3-regulated genes is given in Table 2.

II-5c. Regulation of STAT3 expression
The promoters of both the mouse as well as the human STAT3 gene have been cloned recently, which share a high percentage of homology [80,81]. Both promoters contain a low affinity STAT binding site (SBE), a cAMP-responsive element (CRE) and some GC boxes that possibly bind SP1. The STAT3 promoter is inducible by IL-6 and it is suggested that the expression of STAT3 is auto-regulated by STAT3 in cooperation with an unidentified cAMP-responsive Element-binding protein.

II-5d. The role of STAT3 ser727 phosphorylation
Although only one single phosphorylation site has been found in STAT2 and STAT6, which is a tyrosine phosphorylation site that allows STAT dimerization, additional serine phosphorylation sites have been found in all other vertebrate STATs (reviewed in [63]). STAT1, STAT3 and STAT4 all share a consensus PMSP motif in their C-terminal transactivation domain, in which the serine residue is the target of phosphorylation [1,61,62]. All STAT5s contain a PSP sequence in their C-terminus. Much research in the recent years has been focussed on the role of serine phosphorylation on STAT

transactivation as well as on the signal transduction cascades and kinase(s) the are responsible for STAT serine phosphorylation. For individual STATs, these issues are far from resolved, but an overview of the current understanding towards the cause for and effects of STAT serine phosphorylation will be given here.

Table 2. Genes activated by STAT3.

Type	genes
Acute phase proteins	C-reactive protein
	α1-antichymotrypsin
	α2-macroglobulin
	lipopoly-saccharide (LPS)-binding protein
	tissue inhibitor of metallo-proteinases (TIMP)-1
Transcription factors	junB
	c-Jun
	c-Fos
	Interferon regulatory factor (IRF)-1
	myc
	STAT3
	CCAAT enhancer binding protein (C/EBP)δ
Cell cycle related genes	cyclin D1
	cyclin D2
	cyclin D3
	cyclin E
	cdc25A
	p21
Apoptosis related genes	Bcl-2
	Bcl-xL
Negative feedback	SOCS-1
	SOCS-3
other	Interstitial collagenase
	vasoactive intestinal peptide
	pro-opiomelanocortin
	heat shock protein hsp90
	Oncostatin M
	Interleukin 10
	gp130
	ICAM-1
	fibrinogen

Most information concerning the consequences of serine phosphorylation has been derived from the analysis of C-terminal deletion mutants or STAT ser-ala mutants. Since there are no mice with ser-ala mutations in their STAT loci yet, the available information is limited to transient or stable transfection studies in mammalian cells. The most pronounced effects

of STAT serine phosphorylation have been observed in relation to transcriptional activation. Overexpression of STAT3β, which lacks the C-terminal transactivation domain that includes residue ser727, severely impairs the IL-6-induced STAT3 transactivation [43]. STAT1 and STAT3 ser727ala mutants display a strongly reduced transcriptional activity in response to IFN-γ or IL-6-type cytokines, respectively, both on artificial reporters as well as on the regulation of endogenous genes [61]. Although the C-terminal region of STAT3 functions as an important transcription activation domain, the mechanisms that are involved are poorly understood. Possibly, phosphorylation of the ser727 residue enables association of bridging factors or co-activator proteins which couple DNA-bound STAT3 to the basal RNA polymerase II transcription machinery.
Although the most pronounced effects of STAT3 ser727 phosphorylation are those that enhance gene transcription, repression of STAT3 signaling has also been associated with STAT3 ser727 phosphorylation. Chung et al. reported that transactivation of a STAT3 ser727ala mutant was increased in response to EGF, suggesting that ser727 phosphorylation either prevents tyr705 phosphorylation or increases tyrosine dephosphorylation [82]. Treatment of cells with stress-inducers like UV, which induce STAT3 ser727 phosphorylation via the JNK signal transduction cascade but do not activate Jaks, prior to cytokine stimulation, results in reduced levels of STAT3 tyrosine phosphorylation and transactivation [83]. These data also suggest that STAT3 ser727 phosphorylation negatively regulates STAT3 tyr705 phosphorylation. However, mechanistic explanations are still lacking for these observations and the relative contribution of ser727 phosphorylation to the regulation of STAT3 tyr705 phosphorylation *in vivo* is presently unclear.
The PMSP motif in the C-terminal regions of STAT1, STAT3 and STAT4 resembles the MAPK consensus sequence PxS/TP [84], suggesting that members of the MAPK family participate in STAT serine phosphorylation. Much research in the past few years has focussed on the identification of the kinases that are responsible for STAT serine phosphorylation. However, these issues are far from resolved for individual STAT members. It has now been demonstrated that, depending on the cytokine and cellular setting, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38MAPK (p38) can be involved in mediating STAT1 and STAT3 serine phosphorylation [82,83,85-89]. Direct phosphorylation of STAT1 and STAT3 by ERK, JNK and p38 *in vitro* has been demonstrated in *in vitro* kinase assays. However, the relative contribution of each of these kinases *in vivo* will depend on a variety of factors, including cell-type specific expression of signal transduction components, serine kinases and their affinities for individual STAT members.

II-6. STATs during evolution

The appearance of multi-cellular organisms created the need for efficient cell-cell communications to coordinate suitable responses to environmental changes. The STAT family of transcription factors is involved in both signal transduction events as well as in the regulation of gene expression. Currently, STATs have been cloned from Dictyostelium (slime mold), Drosophila (fly), Anopheles gambiae (fly), C.elegans (worm), Gallus gallus (chicken), Xenopus leavis (frog), Oncorhyncus mykiss (salmon), Danio rerio (zebrafish), Bos taurus (cow), Sus scrofa (pig), Rattus norvegicus (rat), Mus musculus (mouse) and

homo sapiens (human). Possibly, STATs even exist in plants, known as the GRAS family of proteins.

STATs are absent from the yeast genome, but are present in lower eukaryotes as far back as Dictyostelium, in which it mediates the selective expression of an extracellular matrix protein in pre-stalk cells, in response to a differentiation-inducing factor [90-92]. The D-STAT of Drosophila has been implicated in embryonic pattern formation, hemocyte differentiation, sex determination and the regulation of proliferation in imaginal discs [93-95]. The Ag-STAT from Anopheles gambiae is expressed during all developmental stages and is proposed to play a role in immune responses [96]. D-STAT and Ag-STAT are most similar to the vertebrate STAT5 and STAT6 (Fig.6). The XSTAT1 from Xenopus Leavis shares over 90% identity with human STAT1 and in expressed throughout all stages of embryonic development [97]. From Danio rerio, both STAT1 and STAT3 have been cloned, designated as ZSTAT1 and ZSTAT3 [98]. Whereas STAT3 is expressed during embryogenesis in a tissue specific manner, no ZSTAT1 expression was found in the early embryo [98]. Mammalian STATs are widely expressed, are activated in response to cytokines and gene disruption experiments reveal a smaller subset of evidently non-redundant functions. An overview of the phylogenetic distribution of various STATs is given in Fig.5.

Recently, it has been proposed that the GRAS family of plant-specific proteins that play important regulatory roles in diverse aspects of plant development are related to STAT proteins [99]. STATs and GRASs share a similar domain organization, including the presence of a DNA binding domain, a dimerization domain that consists of an SH2 domain and a phospho-tyrosine residue, and possibly a C-terminal transactivation domain including a phospho-serine residue. Like STATs, GRAS proteins act as intracellular intermediates between extracellular ligands and the activation of genes. However, whether GRASs indeed fulfill similar physiological roles as STATs and belong to the same group of transcription factors remains to be elucidated.

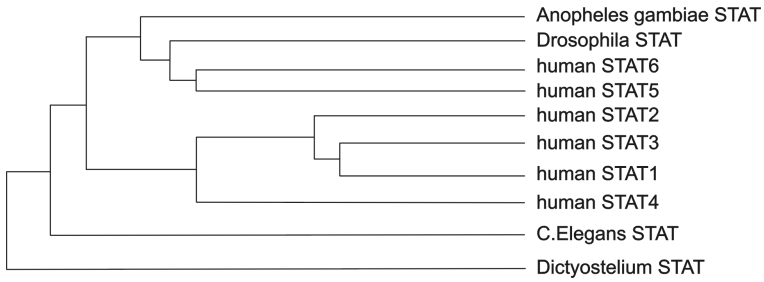

Figure 5. Dendogram based on the alignments of various STAT sequences. (adapted from [100]).

III. Negative regulation of STAT signal transduction

Like all important physiological processes, cytokine-induced STAT activation needs to be precisely controlled both spatially and temporally. In most systems, STAT activation is transient, which has led to the hypothesis that efficient negative feedback mechanisms

must exist. Some of the possible mechanisms that have been considered are (1) upregulation of negative feedback proteins that interfere in the cytokine-induced STAT pathway, (2) activation of tyrosine and/or serine phosphatases, (3) receptor internalization, (4) protein degradation and (5) the expression of nuclear inhibitors of STAT signal transduction (Fig.6).

III-1. Negative feedback proteins: SOCS

Over the past few years, a new family of proteins has been discovered, designated as suppressors of cytokine signaling (SOCS) proteins, also referred to as Jak-binding proteins (JAB) or STAT-induced STAT inhibitors (SSIs) [100-102]. These proteins comprise of a family of negative feedback proteins since their transcription is induced by cytokine stimulation. Currently, this group consists of seven members, SOCS-1-SOCS-7, of which SOCS-1 and SOCS-3 have been studied most extensively [103]. The SOCS proteins share structural similarities including a central SH2 domain, a highly homologous C-terminal region designated as the SOCS-box, and an N-terminal region of variable length and a highly variable amino acid sequence. SOCS-1 inhibits signaling by a wide range of cytokines including LIF, IL-6, IL-4, GH, prolactin, TPO, interferons (particularly IFN-γ), and stem cell factor (kit ligand). Initially, it has been proposed that SOCS-1 binds directly to the kinase domain (JH1) of Jaks (Jak1, Jak2, Jak3 and Tyk2) thus inhibiting kinase activity as well as subsequent Jak-mediated phosphorylation of downstream substrates such as the receptor and STATs [104]. The SH2 domain of SOCS-1 binds to a phosphopeptide corresponding to the activation loop around tyrosine 1007 of Jak2, but an additional stretch of 30 amino acids N-terminal to the SH2 domain is required to enable high affinity binding to Jak2 [105-107]. Both the SH2 domain and the N-terminal region of SOCS-1 are required for inhibitory activity, while the SOCS box appeared to be dispensable [104]. SOCS-3 appears to inhibit many of the same cytokine/receptor systems as SOCS-1, but also downregulates IL-2, IL-3 and leptin signal transduction [108,109]. Although at high concentrations SOCS-3 can interact with Jaks, it has a much lower affinity towards Jaks as compared to SOCS-1 [104]. Rather, SOCS-3 appears to inhibit cytokine signaling by associating with the gp130 receptor directly [110]. Mutation analyses revealed that the phosphotyrosine residue 759 of the gp130 receptor mediates the direct association with SOCS-3, thus preventing ligand-induced receptor and STAT activation. The N-terminal domains of SOCS-1 and SOCS-3 are interchangeable without loss of function, suggesting that both proteins may inhibit JAK kinase activity via their N-terminal domain, and that the SH2 domain either binds Jaks (as is the case for SOCS-1) or the gp130- receptor (as is the case for SOCS-3) [104]. Furthermore, since SHP2 and SOCS-3 associate with the same phosphotyrosine residue at position 759, it is plausible that SOCS-3 also inhibits activation of the RAS/Erk pathway, which is activated by SHP2. Despite the dispensability of the SOCS box for the biological activity of SOCS-1 and –3 in overexpression systems, the conservation of this domain in all SOCS proteins suggest that it does play an important physiological role. It has recently been demonstrated that elongin B and elongin C have a high affinity for the SOCS box and this interaction might serve to recruit a ubiquitin ligase system that targets SOCS proteins to proteolytic degradation by the 26S proteasome [111,112]. Whether this mechanism indeed applies to SOCS proteins *in vivo* to mediate their degradation remains to be elucidated.

III-2. Phosphatases

Another group of proteins that potentially negatively regulate STAT signal transduction are the tyrosine phosphatases (PTPs). However, information is limited on the PTPs that might be involved and the exact physiological roles that PTPs might play *in vivo*. Some progress has been made however, which will be discussed here.
PTPεC was recently identified as a protein-tyrosine phosphatase that inhibits IL-6-induced STAT3 activation, probably by inhibiting Jak activity [113]. Overexpression of wild-type

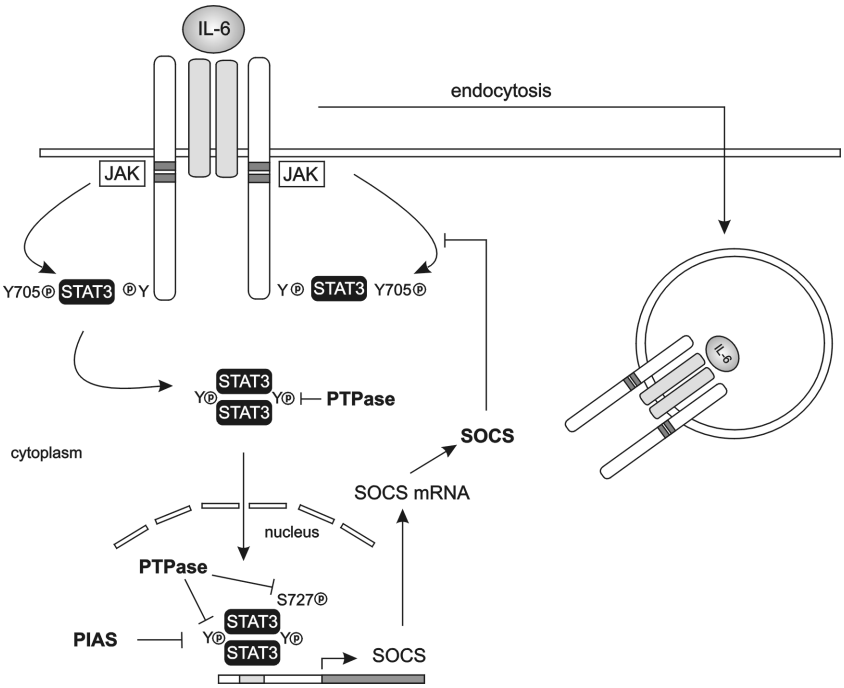

Figure 6. Negative regulation of IL-6-induced STAT signal transduction. Several mechanisms possibly lead to downregulation of IL-6-mediated signals including upregulation of negative feedback proteins (SOCS), activation of nuclear or cytoplasmic phosphatases (PTPases), inhibitory proteins like PIAS, and receptor endocytosis.

PTPεC, but not of an inactive mutant of PTPεC, inhibited the IL-6-induced tyrosine phosphorylation of Jak1, Tyk2 and gp130, and the concomitant tyrosine phosphorylation of STAT3. Also, overexpression of PTPεC severely abrogated the IL-6-induced IRF-1 expression as well as the IL-6-induced terminal macrophage differentiation of M1 cells.
Furthermore, the use of phosphatase inhibitors has led to the hypothesis that PTPs control cytokine-induced STAT signaling since deletion of PTP activity has resulted in constitutive STAT activation in a various cellular settings. In resting cells, the phosphotyrosine content is maintained at less than 1% of the total of phosphoamino acids through the constitutive activities of PTPs. In cells that are briefly exposed to the PTP inhibitor pervanadate, a constitutive phosphorylation of Jak1, Jak2 and Tyk2 was

observed, with the concomitant constitutive activation of STAT1 and STAT3 [114]. Similar observations have been published for STAT6. Moreover, Haspel et al. demonstrated that in cells treated with staurosporin or sodium vanadate, STAT1 is phosphorylated on tyrosine and translocated to the nucleus upon IFN-γ stimulation, but that nuclear export is severely disturbed [115]. These data indicate that a nuclear PTP is required for the inactivation of STAT1 and suggest a model in which activated nuclear STAT1 is (tyrosine) dephosphorylated in the nucleus to allow re-entry of STAT1 monomers to the cytoplasm. However, the PTP involved in this process has currently not been identified. Also, no serine phosphatases have been isolated yet which potentially control serine phosphorylation of STAT3.

III-3. Receptor internalization

Upon the production of cytokine receptors in the endoplasmic reticulum, they are finally translocated to the plasma membrane. However, cytokine receptors are not permanently located at the cell surface, but also undergo endocytosis. This is studied in some detail for the gp130 receptor. After IL-6 binding, the gp130 receptor is efficiently internalized, resulting in a complete depletion of IL-6 surface binding sites within 60 min [116]. Thus, IL-6 down-regulates its own receptor. De novo gp130 protein synthesis is necessary to replenish IL-6 binding sites, suggesting that after internalization, receptor and ligand are degraded. Whether it is the IL-6-induced activation of the JAK-STAT pathway that triggers endocytosis, or possibly dimerization of gp130, is presently unclear. Recent evidence suggests that a dileucine-based internalization motif as well as phosphotyrosine residues might be involved in the association with adaptor proteins which target the receptor towards endocytosis [117]. Indeed, constitutive, ligand-independent endocytosis of gp130 (and LIFR) has been observed that depends in part on dileucine motifs within the cytoplasmic domain of the receptor [117,118]. The adapter protein AP2 associates with gp130 via the dileucine motif, which presumably results in a transfer of the receptor into clathrin-coated pits, followed by efficient endocytosis, trafficking from endosomes to lysosomes, and finally lysosomal degradation [119]. However, more detailed studies in which the internalization rates of gp130 are measured are required to establish the physiological role of receptor internalization as a negative feedback mechanism.

III-4. Protein degradation

It is not very likely that protein degradation is involved in the negative regulation of STAT signaling. In studies using proteasome inhibitors, no effects were found on the turnover of STAT1 and STAT3 [10]. Furthermore, metabolic labeling half-life studies have indicated that at least STAT3 has a rather long half-life (>8 h), which is not reduced upon IL-6 stimulation [10]. Also, the half-life of Jaks has been estimated to extend the kinetics of the transient kinetics of STAT activation. Taken together, these data indicate that protein degradation is not likely to play a role in negatively regulating STAT signal transduction.

III-5. PIAS proteins

Recently a protein inhibitor of activated STAT3 (PIAS3) has been identified, which associates with and prevents DNA binding of tyrosine phosphorylated nuclear STAT3

[120]. A similar protein, PIAS1, has been cloned that specifically inhibits STAT1 DNA binding [121]. PIAS-1 and -3 associate *in vivo* with the N-terminal region of STAT1 and 3 respectively, and are widely expressed in a variety of tissues independent of cytokine stimulation. The physiological importance of these proteins is currently unknown.

IV. STAT interactions with other proteins

Most transcription factors function in multi-protein enhanceosomes to initiate gene transcription. Interactions of STATs have been described with hormone receptors, mini-chromosome maintenance proteins, members of the AP-1 family and members of the IFN regulatory factor (IRF) family [68,122-125]. Also, interactions between some STATs and CREB-binding protein (CBP)/p300 have been described [58,125,126]. The p300 and CBP transcriptional co-activators are important regulators of many cellular processes. They contain histone acetyltransferase (HAT) domains, are involved in chromatin remodeling [127], and interact with a wide range of DNA binding transcription factors, including p53, E2F, AP-1, nuclear receptors, MyoD, the p65 subunit of NF-κB and many others [128,129]. Specifically, p300/CBP has been reported to interact with STAT1, where both the N-terminal as well as the C-terminal region of STAT1 appears to be involved in its association with p300/CBP [125]. Also, p300/CBP interactions with STAT5 have been described [130].
Recently, STAT3-interacting protein (StIP1) has been identified, which might serve as a scaffold protein that promotes the interaction between Jaks and STAT3 [131]. Similar mechanisms might be involved for other STATs as well. Finally, the nuclear protein PIAS associates with activated nuclear STATs and inhibits their transcriptional activities. Specifically, PIAS-1 associates with STAT1 and PIAS-3 associates with STAT3. Recently, the zinc finger protein Gfi-1 has been identified as a new regulatory factor in STAT3-mediated signal transduction [132]. Gfi-1 associates in the nucleus with STAT3, and strongly enhances IL-6-induced STAT3 transactivation. Also, the inhibitory effect of PIAS3 on STAT3 transcriptional activity was overcome by Gfi-1 overexpression, indicating that the balance between PIAS3 and Gfi-1 might play an important regulatory function in STAT signaling.

V. Cross-talk

It is increasingly being appreciated that multiple cytokines act in concert on a single cell *in vivo*, indicating that the combined effects of cytokines might differ from the effects that individual cytokines have on the activation of signal transduction cascades. It has been demonstrated that a considerable amount of cross-talk exists between signaling events initiated by IL-6 in concert with other cytokines or growth factors.

V-1. Negative cross-talk

The expression of SOCS proteins is regulated by a wide variety of ligands, some of which do not activate the JAK-STAT pathway. Although knowledge on how the SOCS promoters are regulated is still limited, to date only the murine SOCS-1 and SOCS-3 promoters have been cloned and partially characterized, some of the involved mechanisms

have been unraveled [133,134]. The SOCS-1 promoter contains binding sites for IRF-1, which expression is induced by activation of STAT1, and the IFN-γ-induced SOCS-1 expression is at least in part mediated via IRF-1 [133]. The murine SOCS-3 promoter contains two STAT1/3 binding sites, which are required for the LIF-induced upregulation of SOCS-3 [134]. However, it is expected that other important regulatory sequences will be identified in the near future that also influence the expression of SOCS-1 and –3.
Part of the negative cross-talk between cytokines and IL-6 involves upregulation of SOCS proteins. Bode et al. demonstrated that LPS and TNFα inhibit STAT3 activation in macrophages by inducing SOCS-3 mRNA, possibly via the p38 MAP kinase [135]. These effects were cell-type specific, since no effects of LPS and TNFα on IL-6-induced STAT3 signaling were observed in liver cells. Similar observations were reported by Decker and colleagues, who show that LPS suppresses IFN-γ-induced STAT1 activation in macrophages by upregulation of SOCS-3 [136]. However, LPS and TNFα might also negatively regulate IL-6 signal transduction by SOCS-independent mechanisms. Ahmed et al. reported that LPS, IL-1 and TNFα inhibited IL-6-induced STAT3 activation in the absence of de novo protein synthesis, indicating that SOCS negative feedback loops are not involved [137]. They show that the membrane-proximal region of the cytoplasmic domain of the gp130 receptor, which does not contain the tyr759 SOCS-3 binding residue, is sensible to the negative effects of LPS, IL-1 and TNFα. However, the involved mechanism still needs to be elucidated.
Furthermore, interleukin-1β (IL-1β) suppresses the IL-6 dependent induction of type II acute-phase response genes in liver cells by a proteasome-dependent mechanism [138]. This mechanism might involve a phosphatase that is activated by IL-1β, since the inhibition could be reversed by treatment with the phosphatase inhibitor sodium vanadate. It was postulated that IL-1β-induced degradation of an unknown protein might lead to the release of a phosphatase that prevents IL-6-induced tyrosine phosphorylation of STAT1. IL-1β did not affect the IL-6-induced activation of STAT3 in this setting.
The involvement of phosphatases has also been implicated in the negative regulation of STAT activation via the ERK/MAPK signal transduction cascade. Pre-stimulation of myeloid, 293T and CHO cells with PMA, ionomycin or GM-CSF strongly reduced IL-6-induced activation of Jaks and STAT3, which did not require do novo protein synthesis [139]. Unfortunately, the phophatase(s) that is involved has not been identified yet.
Estrogens suppress IL-6-induced osteoporosis and the growth of multiple myeloma cells by repressing IL-6 and IL-6 receptor gene expression [140]. Also, a direct interaction between the estrogen receptor (ER) and STAT3 has been demonstrated, which negatively regulates STAT3 transactivation [140]. Although the inhibitory mechanisms of the ER on STAT3 signaling have not been resolved yet, it appears that the ER negatively regulates STAT3 transactivation at the level of transcription initiation, rather than at the level of STAT3 tyr705 phosphorylation or nuclear import.

V-2. Positive cross-talk

Besides negative cross-talk, cooperative effects between IL-6 and other cytokines have also been described. In mice, Bone Morphogenetic Proteins (BMPs) and IL-6-type cytokines synergistically induce astrocyt differentiation in fetal brain [141,142]. Both BMP2 and BMP7 act in concert with either IL-6 or LIF to induce atrocyte differentiation from neuroepithelial cells and BMPs and IL-6 or LIF synergistically upregulate the

expression of glial fibrillary acidic protein (GFAP) [141,143]. The GFAP promoter contains one STAT3 binding site, and probably also SMAD binding sites although these have not been mapped yet, and from reporter assays and *in vivo* differentiation studies it has been shown that STAT3 and SMAD1 are bridged by the co-activator protein p300. This STAT3-SMAD1 complex bridged by p300 is involved in the cooperative signaling of BMPs and IL-6-type cytokines and the subsequent induction of astrocytes from neural progenitors [141].

Since many gene promoters contain binding sites for multiple transcription factors, it is conceivable that the level of gene transcription will depend on the activation status each of the transcription factors involved. Since activation of a specific transcription factor often involves activation its specific receptor complex or upstream signal transduction cascade, cooperation between multiple cytokines is sometimes required to enable high transcription levels of the gene involved. For instance, a number of gene promoters that contain both AP-1 (TRE) and STAT3 (IRE) binding sites have now been identified, including the junB, the vasoactive intestinal peptide, the c-Fos, and the α_2-macroglobulin promoters [144-147]. In these cases, it has been demonstrated that both the STAT3 and AP-1 DNA binding sites contribute to the regulated expression of these genes. Furthermore, the C/EBPδ promoter contains binding sites for both STAT3 and Sp1, and binding of both transcription factors is required to initiate transcription [148]. The c-Fos promoter contains binding sites for serum response factor (SRF) and STAT3, whereby c-Fos expression required activation of both SRF and STAT3 in response to GM-CSF and serum [149].

In some cases however, binding sites for two transcription factors partially overlap. For instance, overlapping binding sites for STAT3 and NF-κB can be found on promoters of several APP genes. IL-1 and IL-6, two early-response cytokines expressed during an acute inflammatory reaction, regulate the expression of several acute phase proteins (APP) in the liver. IL-1 relays its signal to specific genes via NF-κB, whereas IL-6 sends its signal to the nucleus via STAT1 and STAT3. Both STAT3 and NF-κB are active during inflammation and are capable of binding to a STAT3/NF-κB overlapping DNA motif derived from the alpha2-macroglobulin gene promoter although this promoter region can not be occupied by these two transcription factors simultaneously. It has been suggested that these transcription factors regulate each others' function through competition for overlapping DNA binding sites [150].

VI. Biological functions of STAT3

Activation of STATs results in expression of genes that control critical cellular functions including cell proliferation, survival, differentiation and development, as well as specialized functions such as those associated with immune responses. Although the STAT family is highly structurally conserved, there are distinct differences both in primary sequences and function. Here, some of the important biological functions of STAT3 will be reviewed, most of which information has emerged from studies using homozygous deletion or tissue-specific conditional knockout mice.

Unlike other STAT family knockout mice, STAT3 knockout mice are embryonic lethal [151]. Between days 6.5 post coitum (dpc) and 7.5 dpc, the embryos showed rapid degradation. The onset of STAT3 mRNA transcription in wild type mice embryos was exclusively found in visceral endoderm from day 6.0 dpc, which has an important function in metabolic exchange between embryo and maternal blood [151]. The coincidence of the

onset of embryo degradation with STAT mRNA expression suggests that STAT3 knockout embryos die due to the impaired functions of visceral endoderm such as nutritional insufficiency. Since this early embryonic lethality, the role of STAT3 in cytokine-mediated functions could not be assessed in adult tissues. Therefore, STAT3 has been conditionally knocked out in a tissue- or cell-specific manner by Akira and colleagues using the Cre-loxP recombination system (reviewed in [152]). STAT3-deficient T cells displayed a severely impaired proliferative response to IL-6 due to a defect in IL-6 mediated suppression of apoptosis, demonstrating the anti-apoptotic function of STAT3. Furthermore, STAT3-deficient T-cells showed a partial defect in IL-2-induced proliferation, due to impaired IL-2 receptor α chain expression induced by IL-2, whereby STAT3 is probably indirectly involved. Macrophages lacking STAT3 displayed increased inflammatory responses. STAT3-deficient keratinocytes were characterized by an impaired hair cycle and wound healing due to defective keratinocyte motility. Finally, the mammary gland displayed a delayed involution due to impaired epithelial cell apoptosis in the absence of STAT3 expression. STAT3 has not been conditionally knocked out in other tissues yet, but experiments are underway.

Further information has emerged from overexpression studies using dominant negative mutants of STAT3 in various cell types. A role for STAT3 in myeloid differentiation has been described by Nakajima et al., who utilized STAT3 mutants lacking the tyrosine phosphorylation site (tyr705phe) [153]. This mutant blocked IL-6 and LIF-induced macrophage differentiation of M1 myeloid leukemia cells, indicating that STAT3 is required for macrophage differentiation. Fukada et al. have demonstrated that STAT3 also plays an important physiological role in myeloid cell survival [154]. In the IL-6-responsive pro-B cell line BAF-B03, STAT3 plays a key role in the G1 to S phase cell-cycle transition through an upregulation of cyclins D2, D3 and A, and cdc25A, and the concomitant downregulation of the cell-cycle inhibitors p21 and p27. Also, STAT3 signaling is involved in the anti-apoptotic response in this cell line, possibly via upregulation of Bcl-2 [154]. In related studies, Catlett-Falcone et al. showed that autocrine activation of the IL-6/gp130 pathway renders human multiple myeloma cells resistant to Fas-induced apoptosis [155]. This response correlated with a constitutive activation of STAT3 and was blocked by a Jak2 kinase-selective inhibitor, demonstrating a necessary role for STAT3 in the survival mechanism possibly involving the upregulation of the anti-apoptotic protein Bcl-xL.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of pre-implantation embryos, which differentiate into specific cell types that comprise all the three germ-layers of the embryo. Recently, it has been demonstrated that mouse ES cells can be maintained in an undifferentiated state when cultured with LIF, either on gelatin coated tissue culture dishes or on fibroblast feeder cell layers [156-159]. The pluripotency of mouse stem cells depends on LIF-activated STAT3, since mutation of either the gp130 receptor or STAT3 itself abrogated the self-renewal of ES cells and led to the onset of differentiation. Consequently, mouse stem cells can be cultured and kept pluripotent on gelatin coated tissue culture flasks when maintained in medium containing LIF. These data indicate that signals for the suppression of differentiation in ES cells involve STAT3.

Taga and colleagues have demonstrated that LIF and IL-6 induce astrocyte differentiation in synergy with BMP-2 or BMP-7 [141,143]. The expression of glial fibrillary acidic protein (GFAP), which is an astrocyte marker, is regulated by activated Smads and STAT3, which are bridged by the transcriptional co-activator protein p300.

In the liver, IL-6-induced STAT3 activation is required for liver cell regeneration [11,74]. Also, in the acute phase response STAT3 signal transduction has been implicated [160]. In response to hepatocyte growth factor, STAT3 mediates tubulogenesis [161]. In response to VEGF, STATs mediates vasculogenesis [162]. An overview of genes that are regulated by STAT3 is given in Table 2.

VII. The role of STAT3 in oncogenesis

In contrast to normal signaling, in which STAT activation is rapid and transient, constitutive signaling by STATs has been increasingly associated with malignant cell growth. Subversion of a cell's normal genetic program results in alterations in the expression patterns of genes involved in various facets of cellular transformation, such as enhanced cell proliferation, anchorage-independent growth, survival, morphological changes and anti-apoptosis. These processes are now increasingly being understood at the molecular level by functional analyses of key signaling components, including dysregulation of STAT transcription factors. Constitutive STAT activation has been observed in a variety of human cancers, including breast cancers, head and neck cancers, lymphomas and leukemias (Table 3, [155,163-176]). In the normal signaling program, the initial step of STAT activation involves a ligand-induced tyrosine kinase activation, which leads to the phosphorylation of STATs on tyrosine residues. In many cancers, a ligand-independent activation of STATs has been observed, suggesting that a constitutive activation of intrinsic tyrosine kinases might initiate STAT signal transduction in a cytokine-independent manner (Table 4). Indeed, progress has been made on the identification of intracellular oncogenic tyrosine kinases that are capable of activating STATs, which will be discussed here.

The first genetic evidence implicating aberrant STAT activation in oncogenesis was derived from studies in Drosophila. A lethal gain-of-function mutation in the Drosophila JAK homologue, hopscotch, results in hyperactive JAK tyrosine kinase activity causes leukemia-like defects [39]. Dominant suppressors of this phenotype map to loss-of-function mutations in the Drosophila homologue of the mammalian STAT5, suggesting that dysregulation of JAK kinase activity resulting in constitutive activation of D-STAT directly leads to the formation of hematological malignancies in fruit flies [94,177].
The first evidence for the involvement of aberrant JAK activation in human cancers came from a chromosomal translocation (9;12) implicated in some acute lymphoid leukemias (ALL) [178-180]. This translocation results in a fusion protein that contains the kinase domain of Jak2 fused to the oligomerization domain of the Ets transcription factor (Tel-Jak2) and possesses constitutive tyrosine kinase activity. Introduction of the Tel-Jak2 fusion protein into human hematopoietic cell lines results in constitutive activation of STAT1, STAT3 and STAT5 and induces a ligand-independent proliferation. Mice expressing Tel-Jak2 develop myelo- and lympho-proliferative diseases.
The oncogenic Src tyrosine kinase induces a constitutive activation of STAT3 in the absence of cytokine stimulation [181]. Cellular transformation of NIH3T3 cells induced by v-Src is blocked by co-expression of dominant negative forms of STAT3, indicating that constitutive activation of STAT3 is one of the possible pathways that lead to

malignant transformation [182-185]. Since then, several other oncoproteins of the receptor and non-receptor tyrosine kinase family have been identified which activate STATs. Src-family members such as Lck and v-Fps (Fes) activate STAT3 and STAT5 in T cells and fibroblasts, respectively [172,186,187]. Oncoproteins such as v-Sis, v-Ros, insulin-like growth factor I (IGF-1) receptor, and c-Eyk/v-Eyk have been shown to activate predominantly STAT3 in fibroblast cell lines and to a lesser extend STAT1 (reviewed in [163]). In epithelial cells, the cellular tyrosine kinase Etk/BMX is able to activate STAT1, STAT3 and STAT5 [163]. In chronic myeloid leukemia, the oncogenic fusion protein BCR-Abl has been shown to activate STAT5, which is essential for cellular transformation [170,188,189].

Table 3. Activation of STATs in human tumors and cell lines.
(modified version from refs [167] and [168]).

Tumor type	Activated STAT
Leukemias (tumors and cell lines)	
Human T-cell lymphothropic virus dependent	STAT3, STAT5
Erythroleukemia	STAT1, STAT5
Acute lymphocytic leukemia (T-cell)	STAT1, STAT5
Acute lymphocytic leukemia (B-cell)	STAT1, STAT5
Acute myelocytic leukemia	STAT1, STAT3, STAT5
Chronic myelocytic leukemia	STAT5
Chronic myelo-monocytic leukemia	STAT1, STAT5
Chronic lymphocytic leukemia	STAT1, STAT3 (*)
Megakaryocytic leukemia	STAT5
Lymphoma (tumors and cell lines)	
EBV-related Burkitt's lymphoma	STAT1, STAT3
Mycosis fungoides	STAT3
Herpesvirus saimiri-dependent T-cell	STAT1, STAT3
LSTRA cell line (T cells)	STAT3, STAT5
Cutaneous T cell lymphoma	STAT3, STAT5
Other cancers (tumors and cell lines)	
Renal cell carcinoma	STAT3
Prostate carcinoma	STAT3
Melanoma	STAT3
Prancreatic adenocarcinoma	STAT3
Ovarian carcinoma	STAT3
Breast cancer (tumors)	STAT1, STAT3
Breast cancer (cell lines)	STAT3
Head and neck cancer	STAT1, STAT3
Multiple myeloma (tumors and cell lines)	STAT1, STAT3

(*) only constitutive STAT3 serine phosphorylation

While in some cases constitutive STAT1 activation has been detected in human tumors, dysregulation of STAT3 and STAT5 has been observed most frequently. It appears unlikely that STAT1 contributes to oncogenesis since activation of STAT1 has been associated with growth inhibitory effects in most cellular settings. In addition, homozygous STAT1 knockout mice are predisposed to certain malignancies and develop tumors with more rapid frequency and kinetics than wild-type littermates [190]. Possibly, the growth inhibitory effects of activated STAT1 can be overcome by the simultaneous activation of STAT3 and STAT5, whereby the pro-proliferative activities of STAT3 and STAT5 might predominate over the anti-proliferative activity of STAT1.

Table 4. Activation of STATs by various oncogenes.
(adapted from [167]).

Cell Type	Oncogene	Activated STAT
Fibroblast	v-Src	STAT3
	c-Src	STAT3
	v-Fps (Fes)	STAT3
	v-Sis (PDGF)	STAT3
	Polyomavirus middle T antigen	STAT3
	SV40 large T antigen	-
	v-Ras	-
	v-Raf	-
	v-Ros	STAT3
	IGF-I receptor	STAT3
	c-Eyk	STAT1, STAT3
	v-Eyk	STAT1, STAT3
Pre-B lymphocytes	v-Abl	STAT1, STAT5
Galbladder adenocarcinoma	v-Src	STAT3
Hepatoma	HBx	STAT3, STAT5
Mammary/lung epithelial	v-Src	STAT3
	Etk/BMX	STAT1, STAT3, STAT5
Basophil/mast cells	BCR-Abl	STAT1, STAT5
Erythroleukemia/blast cells	BCR-Abl	STAT1, STAT5
Myeloid	v-Src	STAT1, STAT3, STAT5
	v-Fgr	-
Primary bone marrow	BCR-Abl	STAT5
	v-Mos	-

Direct evidence for a causal role of STAT3 in oncogenesis arose from experiments performed by Bromberg et al., who generated a constitutive active mutant of STAT3 by substitution of two cysteine residues within the C-terminal loop of the SH2 domain (STAT3-C) [191]. This mutant dimerizes spontaneously, binds to DNA, and activates transcription in the absence of ligand stimulation. The STAT3-C molecule causes cellular transformation of immortalized fibroblasts and tumor formation in nude mice. The events downstream of STAT3 that promote tumorgenesis are still unclear, but might include enhancing effects on cell cycle progression via upregulation of cyclin D1 and/or providing protection against apoptosis by upregulation of Bcl-xL.

Scope of this thesis

In response to IL-6-type-cytokines STAT3 is phosphorylated on both tyrosine 705 as well as on serine 727. In chapters 2 and 3 of this thesis it was investigated which signal transduction cascade is involved in IL-6-induced STAT3 ser727 phosphorylation. It could be demonstrated that neither the MAPK/ERK pathway nor the p38/MAPK pathway is involved. In chapter 2 the direct gp130-downstream signal transduction components involved in IL-6-induced STAT3 ser727 phosphorylation could be identified, being the GTP nucleotide exchange factor Vav, the small GTPase Rac-1, and the kinases MEKK-1 and SEK-1/MKK-4. In chapter 3 it is demonstrated that PKCδ is directly involved in IL-6-induced STAT3 ser727 phosphorylation. PKCδ signals downstream of the Vav-Rac-1-MEKK-SEK-1/MKK-4 signal transduction cascade. A model is presented in which activated PKCδ dissociates from SEK-1/MKK-4 and translocates to the nucleus where it associates with and phosphorylates STAT3.

In chapter 4 the role of STAT3 ser727 phosphorylation in the initiation of gene transcription was investigated. It could be demonstrated that the C-terminal 65 amino acids of STAT3 function as an independent transcription activation domain, particularly when a negative charge was introduced at position 727 by mutation of serine into aspartic acid. Evidence is provided that p300 associates with ser727 phosphorylated STAT3 thus enhancing its transcriptional activity. Many transcription factors interact with other proteins, which might introduce specificity of gene expression or influence the levels of mRNAs that are produced. In chapter 5 it is demonstrated that c-Jun and c-Fos cooperate with STAT3 in transactivation of the IL-6-Response Element.

Constitutive activation of STAT3 has been observed in a variety of human cancers. In chapter 6 the activation patterns of STAT3 were investigated in acute myeloid leukemia cells. In 25% of the investigated patients, STAT3 is constitutively activated. This observation could be correlated with a strongly enhanced IL-6 secretion of these cells. Interfering with anti-IL-6 neutralizing antibodies abolished the constitutive STAT3 activation patterns and restored the IL-6 inducibility of STAT3.

In chapter 7, STAT3 signal transduction was investigated in cells that express the oncogene MEN2A-RET. In patients with the inherited MEN2A-syndrome, mutations in the receptor tyrosine kinase RET result in a constitutive and ligand-independent activation

of the MEN2A-RET receptor. It is demonstrated that STAT3 is activated by the MEN2A-RET receptor via two tyrosine STAT3 docking sites, tyr752 and tyr928. Stable NIH-3T3 cell lines expressing both MEN2A-RET and STAT3α but not STAT3β, are characterized by enhanced proliferation and cyclin-D1 promoter activity, and enhanced growth in soft agar. These data indicate that malignant cell growth induced by MEN2A-RET involves its activation of STAT3.

The pluripotency of mouse stem cells depends on LIF-activated STAT3, since mutation of either the gp130 receptor or STAT3 itself abrogates the self-renewal of ES cells and lead to the onset of differentiation. Consequently, mouse stem cells can be cultured and kept pluripotent on gelatin coated tissue culture flasks when maintained in medium containing LIF. In contrast, human stem cells still depend on feeder cells in order to maintain their pluripotency. The experiments in chapter 8 were designed to investigate which signal transduction cascades are activated in response to LIF in human versus mouse embryonic carcinoma (EC) cells (P19 EC versus Ntera/D1 EC). LIF induces both ERK as well as STAT3 activation in mouse P19 EC cells, but LIF-induced proliferation depends only on ERK activity. In contrast, LIF does not activate STAT3 or ERK in human Ntera/D1 EC cells, although receptor components are properly expressed. Possibly, LIF signaling is disturbed in human EC cells due to elevated levels of SOCS-1 expression.

In chapter 9 a summary of this thesis is given in which the results are discussed.